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Characterization of the Human 36-kDa Carboxyl Terminal LIM Domain Protein (hCLIM1)

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Abstract We characterized a human cDNA clone encoding a 36-kDa carboxyl terminal LIM domain protein with a PDZ domain at the amino terminal. This full-length cDNA clone has a predicted open reading frame (ORF) of 329 amino-acid residues. The ORF of this cDNA encodes the human homolog of rat CLP36, and the putative protein is named human 36-kDa carboxyl terminal LIM domain protein (hCLIM1, nomenclature approved by the HUGO/GDB Nomenclature Committee). The hCLIM1 probe was used to hybridize with poly(A)$^+$ RNA of various human tissues. Strong signals were detected in heart and skeletal muscle; moderate signals were detected in spleen, small intestine, colon, placenta, and lung; weaker levels were detected in liver, thymus, kidney, prostate, and pancreas; and no observable signals were detected in brain, testis, ovary, and peripheral blood leukocytes. The hCLIM1 gene was studied by fluorescence in situ hybridization (FISH), somatic cell hybrid analysis, and radiation hybrid mapping, and it is located at the human chromosome 10q26. J. Cell. Biochem. 72:279–285, 1999.

Key words: heart cDNA; CLP36; zinc finger protein; PDZ domain; 10q26; radiation hybrid mapping

The LIM domain is a double zinc finger domain with a consensus sequence of $CX_2CX_{17-19}HX_2CX_2CX_{16-20}CX_2C/D/H$. This domain was first identified in the gene products of *lin-11* from *Caenorhabiditis elegans*, *isl-1* from rat, and *mec-3* from *Caenorhabiditis elegans*. Though implicated by the presence of zinc fingers, the LIM domain is thought to be involved in protein-protein interactions, instead of DNA-protein interactions [Feuerstein et al., 1994; Schmeichel and Beckele, 1994]. LIM domain proteins are found in a diverse range of species and were shown to have a broad spectrum of functions [Dawid et al., 1995; Sanchez-Garcia and Rabbitts, 1994; Taira et al., 1995]. LIM domain proteins can be divided into three main classes: homeodomain proteins, LIM-only proteins, and carboxyl terminal LIM domain proteins [Dawid et al., 1995]. Carboxyl terminal LIM domain proteins contain 1–5 LIM domains, clustered at the carboxyl terminal of the proteins [Dawid et al., 1995; Taira et al., 1995]. Many carboxyl terminal LIM domain proteins have functions related to the cytoskeleton and signal transduction pathway [Dawid et al., 1995; Kuroda et al., 1996]. Carboxyl terminal LIM domain proteins that contain a PDZ domain at the amino terminal were recently identified [Xia et al., 1997]. The members of this subclass of carboxyl terminal LIM domain proteins include actinin-associated LIM protein (ALP), reversion-induced LIM protein (RIL), and a carboxyl terminal LIM domain protein of 36 kDa (CLP36) [Xia et al., 1997]. PDZ is an 80–120 amino-acid domain first identified in the postsynaptic protein, PSD95 [Cho et al., 1992], the *Drosophila* septate junction protein Disc-large [Woods and Byrant, 1991], and the epithelial tight junction protein ZO1 [Willot et al., 1993]. It has been demonstrated that PDZ domains are found in a

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variety of proteins that interact with the cytoskeleton [Ponting and Phillips, 1995]. Some PDZ proteins recognize proteins containing the consensus carboxyl-terminal tripeptide motif S/TXV [Cabral et al., 1996], while others are capable of forming homophilic dimers, as the PDZ domain of nitric oxide synthase binds to the PDZ domain of PSD95 [Brenman et al., 1996].

A human heart cDNA sequencing project was initiated by our group with the aim of understanding the expression profile of transcripts in the heart and discovering novel transcripts expressed in the heart [Hwang et al., 1997]. Throughout this project, several novel transcripts were identified. Furthermore, several novel human LIM domain proteins were identified. They include hCRHP, which is developmentally regulated in the heart [Tsui et al., 1994], FHL1 and FHL2, which contain four and a half LIM domains [Chan et al., 1998; Lee et al., 1998], and hCRP2, which is thought to be a candidate for leukemic translocation [Tsui et al., 1996]. In this study, we characterized another cDNA of a novel human LIM domain protein, hCLIM1, which was found to be the human homolog of CLP36. CLP36 was isolated from rat hepatocytes, and expression of CLP36 was shown to be downregulated when chemical hypoxia was induced [Wang et al., 1995].

MATERIALS AND METHODS

Sequence Analysis of hCLIM1

A human cardiomyopathic heart cDNA library was constructed unidirectionally with the expression vector lambda ZAP (Stratagene, La Jolla, CA), as described previously [Hwang et al., 1995]. Partial sequencing of the cDNA clones isolated from the cDNA library was conducted as described previously [Hwang et al., 1994; Liew, 1993; Liew et al., 1994; Tsui et al., 1995]. Sequences obtained were compared with the GenBank and EMBL nucleotide and protein databases through the NCBI WWW server, using BLASTN and BLASTX searches [Altschul et al., 1990]. One of the cDNA clones exhibited DNA sequence similarity to that of CLP36, but lacked the translation initiation site at the 5′ end of the ORF. By nucleotide sequence homology search using the BLAST electronic mail server, one clone (GenBank accession no. AA065047), isolated from a human adenocarcinoma cDNA library, was found to contain the complete ORF with the 5′ untranslated region. This cDNA clone of hCLIM1, cloned in pBluescript SK^-, was purchased from I.M.A.G.E. Consortium (Rockville, MD) and was used for sequence analysis. The complete sequence of the cDNA of hCLIM1 was determined by primer walking, using dideoxy termination cycle sequencing with a combination of vector and insert-specific primers and the ThermoSequenase cycle sequencing kit (Amersham, Buckinghamshire, UK) and with the use of the A.L.F. automated sequencer (Pharmacia Biotech, Uppsala, Sweden). The amino-acid sequence of the putative protein was predicted using the software DNASIS (Hitachi, Japan).

Subcloning, Expression, and Purification of hCLIM1

Polymerase chain reaction (PCR) of the hCLIM1 clone was performed using a primer specific for the 5′end of the hCLIM1 ORF (5′ TAG GCC *GAG CTC* ACC ACC CAG CAG ATA GAC CTC) and a primer specific for the 3′ untranslated region of hCLIM1 (5′ TAG GCC *GTC GAC* CTG GAG AAC AGT GGT CAC ATC). Both primers contained an end clamp (TAG GCC) at the 5′ end to facilitate cleavage of restriction enzymes. A *Sac*I site was present at the 5′ primer, and a *Sal*I site at the 3′ primer. After digestion with *Sac*I and *Sal*I, the PCR fragment was subcloned into the expression plasmid pQE30 (Qiagen, Chatsworth, CA). The recombinant plasmid pQE30-hCLIM1 was transformed into *Escheriscia coli* strain M15(pREP4). Induction of expression of 6xHis-tagged hCLIM1 protein with 1 mM IPTG was performed. The bacterial cell pellet was resuspended in buffer B (8 M urea, 0.1 M NaH_2PO_4 (2 H_2O, 0.01 Tris, pH 8.0). The supernatant was mixed with Ni-NTA resin (Qiagen) and incubated at room temperature for 2 h. The resin mixture was poured into a column and settled. The resin was washed with twice the volume of buffer C (8 M urea, 0.1 M NaH_2PO_4 (2 H_2O, 0.01 Tris-HCl, pH 6.3), and the desired protein was eluted with buffer C with 100 mM EDTA. The bacterial extract and eluted protein were electrophoresed on a 15% SDS-polyacrylamide gel.

Northern Blot Analysis of Tissue Distribution of hCLIM1

For the determination of tissue distribution of hCLIM1 mRNA, radioactive probes made from PCR products of hCLIM1 were used to perform Northern hybridization with poly(A)$^+$

RNA of various human tissues. The human multiple-tissue Northern blots were purchased from Clontech (Palo Alto, CA). The integrity of the poly(A)$^+$ RNAs of the blots were examined by denaturing gel electrophoresis and normalized with a radioactively labeled human β-actin cDNA control probe (unpublished data, quality control sheet supplied by Clontech). A radioactively-labeled, random-primed probe was made by using the purified PCR product of hCLIM1 as template. The blot was prehybridized for 30 min and hybridized for 1 h at 68°C, using ExpressHyb hybridization solution (Clontech). Membranes were then washed in 2 × SSC with 0.05% SDS twice and again in 0.1 × SSC with 0.1% SDS at room temperature to remove any nonspecific annealing. Autoradiography was performed at -70°C for 24 h.

Chromosomal Mapping of hCLIM1 by Fluorescence In Situ Hybridization, Somatic Cell Hybrid Analysis, and Radiation Hybrid Mapping

The chromosomal location of hCLIM1 was determined by means of fluorescence in situ hybridization (FISH), somatic cell hybrid analysis, and radiation hybrid mapping. FISH analysis was performed as previously described [Garcia-Barcelo et al., 1997], with the pBluescript SK$^-$-hCLIM1 plasmid biotinylated as probe. For somatic cell hybrid analysis, PCR was applied on the monochromosomal NIGMS Human/Rodent Somatic Cell Hybrid Panel 2 (Coriell Institute, Camden, NJ) consisting of 24 genomic DNAs from the same number of human-on-rodent somatic cell lines containing a single human chromosome each plus three control DNAs (human, Chinese hamster, and mouse) [Drwinga et al., 1993], as previously described [Garcia-Barcelo et al., 1997, 1998]. Several pairs of primers specific for the 3′ untranslated region were designed in order to obtain a human-specific band of PCR product that could be differentiated from the rodent background amplification products. The specific pair of primers successfully used was: 5′ ACT TTG GTT TTC CCT CTG CTT GTA AA and 5′ AAA AAC AAA ATC AGT GTC AGA CCC GT. Cycling conditions were: 94°C, 5 min; 35 cycles of 95°C, 50 sec; 55°C, 50 sec; 72°C, 1 min 30 sec; followed by a final extension of 72°C, 10 min. The expected size of the PCR product was approximately 250 bp. The pair of primers described above was used for radiation hybrid mapping, which was performed on the Genebridge 4 Whole-Genome Radiation Hybrid Panel (Research Genetics, Huntsville, AL) consisting of 93 genomic DNAs from the same number of human-on-hamster somatic cell lines, plus two control DNAs (HFL human, A23 hamster) [Gyapay et al., 1996], as previously described [Garcia-Barcelo et al., 1997].

RESULTS

Sequence Analysis of hCLIM1 cDNA

Sequence determination of the cDNA revealed that it was composed of 1,496 bp, encoding a 329-amino-acid protein. The putative protein encoded by this cDNA was named human 36-kDa carboxyl terminal LIM protein (hCLIM1). The nucleotide sequence data have been submitted to the GenBank/EMBL Data Libraries under the accession number U90878. When the ORF of hCLIM1 was compared to that of CLP36, the nucleotide sequences showed 85% homology (data not shown), while the amino-acid sequences showed 87.8% homology (Fig. 1). This suggested that hCLIM1 is the human homologue of CLP36 protein. The calculated molecular weight of the putative hCLIM1 protein is 36.2 kDa. The estimated pI of hCLIM1 is 6.87, as determined by the software GeneWorks 2.5.1. (Oxford Molecular Group Inc., Campbell, CA). A database homology search with BLAST indicated that hCLIM1 shared high homology with other carboxyl terminal LIM domain proteins including ALP, RIL, Enigma, and ENH. The PDZ domain of hCLIM1 shares 54%, 61%, 51%, and 52% amino-acid identity with the PDZ domains of ALP, RIL, Enigma, and ENH, respectively (Fig. 2).

Subcloning, Expression and Purification of hCLIM1 in *E. coli*

hCLIM1 cDNA was successfully amplified using a pair of tailor-made cloning primers. The success of the directional cloning was proved by restriction digestion of the putative recombinant plasmid, PCR, and automated sequencing. After resolving the crude bacterial extract and purified protein in a 15% SDS-polyacrylamide gel, it could be shown that the intense band at about 36 kDa is the 6xHis-tagged hCLIM1 protein. It is only present upon IPTG induction and is retained after purification (Fig. 3). The size of the expressed protein is consistent with the calculated molecular weight of the 6xHis-tagged hCLIM1, which is approximately 36 kDa.

Fig. 1. Comparison of the amino-acid sequences of hCLIM1 and CLP36. Amino acids that are identical are marked by a colon, and those that are similar are marked by a dot. The PDZ domains are in bold, and the LIM domains are underlined.

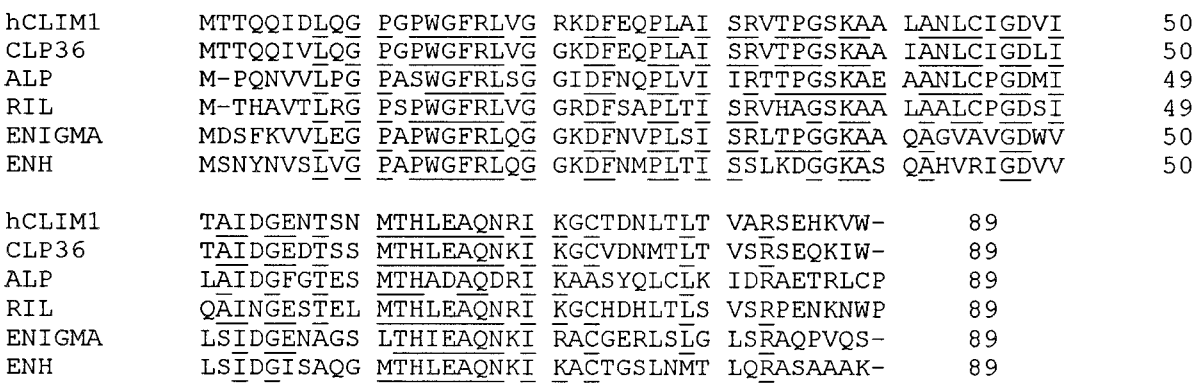

Fig. 2. Multiple sequence alignment of the amino terminals of hCLIM1, CLP36, ALP, RIL, Enigma, and ENH. The consensus amino-acid residues are underlined.

Northern Blot Analysis of hCLIM1

As revealed by multiple-tissue Northern blot analysis using a radiolabeled hCLIM1 probe, hCLIM1 is shown to be expressed in various human tissues. The expression of hCLIM1 is most abundant in heart and skeletal muscle, moderate in spleen, small intestine, colon, placenta, and lung, low in liver, thymus, kidney, prostate, and pancreas, and not detectable in brain, testis, ovary, and peripheral blood leukocytes (Fig. 4).

Chromosomal Mapping of hCLIM1

For FISH mapping of hCLIM1, although the hybridization signal was detected on the q telomeric region of chromosome 10, 10q26, in 50% of the metaphase screened (data not shown), the result was inconclusive due to the presence of background signals. To corroborate the FISH data, somatic cell hybrid analysis and radiation hybrid mapping were performed.

For somatic cell hybrid analysis, human genomic DNA (Human Line IMR91) and DNA

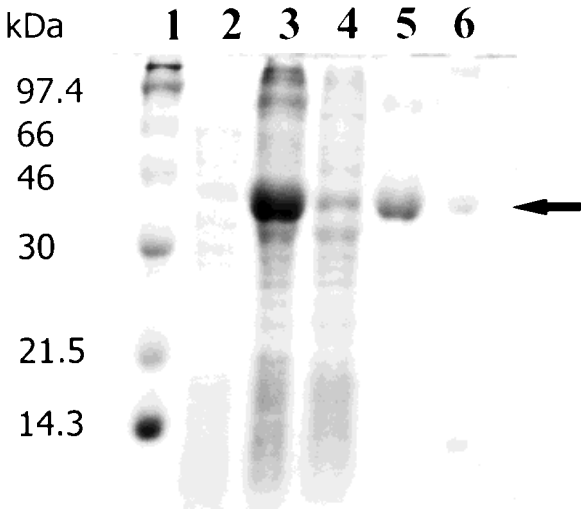

Fig. 3. Expression of recombinant hCLIM1 protein in *E. coli.* SDS-PAGE of 6xHis-tagged hCLIM1 on 15% polyacrylamide gel. **Lane 1,** molecular size marker; **lane 2,** uninduced recombinant bacterial crude extract; **lane 3,** induced bacterial crude extract; **lane 4,** flow through of the Ni-NTA purification column; **lane 5,** protein retained after purification; **lane 6,** protein retained after dialysis. Arrow indicates the 6xHis-tagged hCLIM1 protein.

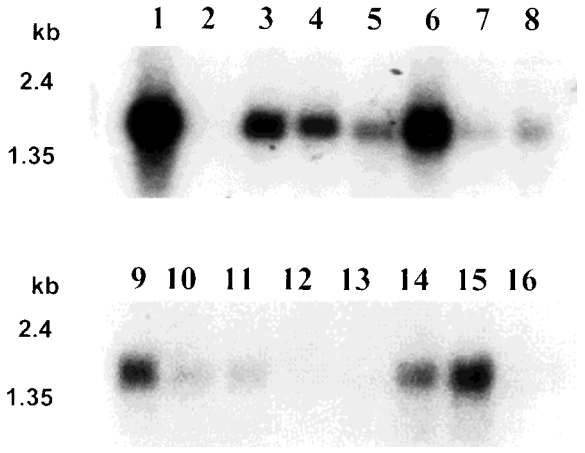

Fig. 4. Northern hybridization of hCLIM1 in human tissues. Two human multiple-tissue Northern blots (Clontech, Palo Alto, CA) with 2 μg of total poly(A)$^+$ RNA of each of the tissues were hybridized with ^{32}P-labeled probe from the coding region of hCLIM1 cDNA. **Lane 1,** heart; **lane 2,** brain; **lane 3,** placenta; **lane 4,** lung; **lane 5,** liver; **lane 6,** skeletal muscle; **lane 7,** kidney; **lane 8,** pancreas; **lane 9,** spleen; **lane 10,** thymus; **lane 11,** prostate; **lane 12,** testis; **lane 13,** ovary; **lane 14,** small intestine; **lane 15,** colon; **lane 16,** peripheral blood leukocytes.

from the somatic cell hybrid line NA10926B, which retained only the human chromosome 10, yielded a single 250-bp band. No 250-bp PCR product was detected from the rest of the hybrids retaining other human chromosomes and rodent DNA. This result implies the location of hCLIM1 to be on chromosome 10. The precise location of hCLIM1 was determined with radiation hybrid mapping. For radiation hybrid mapping, the following data vector was generated from the score of the presence or absence of a 250-bp amplification product in the samples:

00000000100000100010011000001111000001000000001000000000010000011010000001000000000000000000000000.

These data were submitted to the Whitehead Institute/ MIT Center for Genome Research STS Mapping Server (Cambridge, MA). Using a LOD threshold of >21, we were able to establish linkage of hCLIM1 to chromosome 10. Furthermore, the data placed the hCLIM1 gene at 30.11 centiRays (cR) (LOD 3) below framework marker WI-4197 (D10S1593), which maps 665.30 cR from the top of the Chr10 linkage group, placing hCLIM1 at about 695.41 cR from the top telomere of the short arm. This distance of 695.41 cR also placed hCLIM1 as the most distal gene found on chromosome 10. The precise order of the markers is shown in Figure 5.

The FISH mapping data correlate well with the data obtained from radiation hybrid mapping.

DISCUSSION

This report describes the partial characterization of a cDNA-encoding hCLIM1 protein. The amino-acid sequence of hCLIM1 shared high homology to that of the rat CLP36 protein, suggesting hCLIM1 to be the human homolog of CLP36. As a human homolog of CLP36, hCLIM1 protein contains a PDZ domain at the amino terminal and a LIM domain at the carboxyl terminal. This would add hCLIM1 to the family of carboxyl terminal LIM domain proteins with PDZ domains.

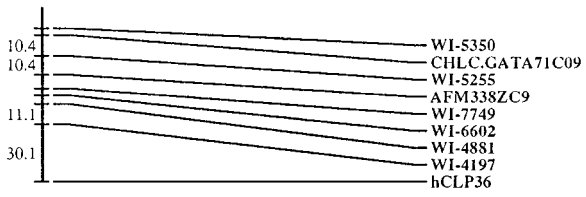

Fig. 5. A partial RH map of human chromosome 10. Using a LOD threshold of >21, the data placed the hCLIM1 gene at 30.11 centiRays (cR) (LOD >3) below framework marker WI-4197 (D10S1593), placing hCLIM1 at about 695.41 cR from the top telomere of the short arm.

Besides sharing homology with its homolog, CLP36, a database homology search with the BLAST program indicated that hCLIM1 shared high homology with other carboxyl terminal LIM domain proteins including ALP, RIL, Enigma, and ENH. While hCLIM1, CLP36, and RIL contain only one LIM domain at the carboxyl terminal, Enigma and ENH have three LIM domains. The sequence homology would suggest that these proteins belong to the same family of carboxyl terminal LIM domain proteins with amino terminal PDZ domains. Apart from ALP, RIL, and CLP36, as suggested by Xia et al. [1997], hCLIM1, Enigma, and ENH should also be included in this family of proteins.

When the expression pattern of hCLIM1 mRNA was compared to that of CLP36 [Wang et al., 1995], interesting results were seen. Although the expression pattern of hCLIM1 in heart, spleen, kidney, testis, and brain corresponds to that of CLP36, the expression pattern of hCLIM1 in skeletal muscle, liver, and lung differs from that of CLP36. CLP36 was shown to express abundantly in liver and lung, and moderately in skeletal muscle, while hCLIM1 was shown to express abundantly in skeletal muscle, moderately in lung, and at lower levels in liver. This difference in expression pattern may be due to the difference in function of CLP36 and hCLIM 1 in humans and rats, but it could also be due to differences in the age and sex of the pool of subjects chosen for the organs.

Our results on mapping of hCLIM1 by FISH and radiation hybrid panel placed the hCLIM1 gene at chromosome position 10q26. Within this region, genes for human ADAM 8 and 12, acyl-CoA dehydrogenase, fibroblast growth factor receptor, uroporphyrinogen-III synthase, and ornithine aminotransferase have been identified. Except for ornithine aminotransferase, deficiency of which would cause gyrate atrophy of the choroid and retina, no disease loci have been mapped to this region.

At present, the physiological role and the importance of hCLIM1 are still unknown. By possessing a PDZ domain and an LIM domain, an implication of the involvement of hCLIM1 in protein-protein interactions is made. Other members of this LIM domain protein family (e.g., ALP, Enigma, ENH, and RIL) were shown to be involved in signal transduction and the cytoskeletal network. ALP is a protein that is expressed in highly differentiated skeletal muscle. It associates with α-actinin 2 at the Z lines of myofibers. The association of ALP to α-actinin 2 occurs via the PDZ domain of ALP and the spectrin-like motifs of α-actinin 2 [Xia et al., 1997]. Enigma was identified as an insulin receptor-binding protein and it may also be involved in mitogenic signaling via its interaction with Ret/ptc 2 [Durick et al., 1996; Wu and Gill, 1994; Wu et al., 1996]. ENH is a protein kinase C interacting protein [Kuroda et al., 1996]. When RIL was isolated from transformation revertant rat cells, the expression of RIL was shown to be downregulated in transformed rat cells and reversed in revertants [Kiess et al., 1995]. In addition, in carboxyl terminal LIM domain proteins with PDZ domains, the PDZ domain and LIM domain were shown to interact with each other, and this interaction may cause the protein to form a multiple protein complex with other proteins [Cuppen et al., 1998]. All these experimental results would lead to the speculation that hCLIM1 may be involved in a signal transduction pathway or the cytoskeletal network through interaction with other proteins, forming a multiple protein complex.

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